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(54) Title: PRODUCTION OF KALLIKREIN (57) Abstract A recombinant human kallikrein having one or more of the biological properties associated with mammalian kallikreins and is characterized by being the product of procaryotic or eucaryotic host expression of an exogenous DNA. The novel kallikrein polypeptide products are useful as vasodilators and in the treatment of male infertility.		

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PRODUCTION OF KALLIKREIN

5 The present invention relates to novel recombinantly
derived glandular (tissue) kallikrein polypeptides
and to methods for producing such polypeptides. The
invention further relates to pharmaceutical
compositions containing such polypeptides and to the
10 use of such polypeptides and compositions as
vasodilators and in the treatment of hypertension
and male infertility.

Background of the Invention

15 Kallikreins are members of a closely related
subfamily of serine proteases. Kallikreins are
characterized by their ability to release vasoactive
peptides, kinins, from kininogen, although the
physiological significance of proteolytic actions of
20 these enzymes seems to be unrelated to the release
of kinins at least in some instances, and certain
kallikreins are thought to be involved in the
specific processing for the generation of
biologically active peptides as well as factors from
25 their precursors. [Fukushima et al., Biochemistry
24, 8037-8043 (1985)].

The cDNA sequences for mouse and rat kallikrein were
isolated from a submaxillary gland and a pancreatic
30 cDNA bank. [Nakanishi et al., Biotechnology 3,
1089-1098 (1985)]. cDNA clones for human kallikrein
were isolated from a human pancreatic cDNA bank
[Fukushima et al., supra] and from a human kidney
cDNA bank, [Baker et al., DNA 4, No. 6, 445-450
35 (1985)]. It was reported that the active enzyme
form consists of 238 amino acids and is preceded by

a signal peptide and profragment of 24 amino acids. It was also noted that the key amino acid residues required for serine proteinase activity (His-41, Asp-96, Ser-190) and for the kallikrein type cleavage specificity (Asp-184) are retained in the human kallikrein as they are in mouse and rat kallikreins.

Summary of the Invention

In accordance with the present invention, a novel class of glandular kallikrein polypeptides is provided. These biologically active kallikrein polypeptides have the amino acid sequence extending from the N-terminus of the formula (I):

3

$$\left(\text{Ala Pro Pro Ile Gln Ser Arg} \right)_n$$

10 20
 +1
 Ile Val Gly Gly Trp Glu Cys Glu Gln His Ser Gln Pro Trp Gln Ala Ala Leu Tyr His

30 40
 Phe Ser Thr Phe Gln Cys Gly Gly Ile Leu Val His Arg Gln Trp Val Leu Thr Ala Ala

50 60
 His Cys Ile Ser Asp Asn Tyr Gln Leu Trp Leu Gly Arg His Asn Leu Phe Asp Asp Glu

70 80
 Asn Thr Ala Gln Phe Val His Val Ser Glu Ser Phe Pro His Pro Gly Phe Asn Met Ser

90 100
 Leu Leu Glu Asn His Thr Arg Gln Ala Asp Glu Asp Tyr Ser His Asp Leu Met Leu Leu

110 120
 Arg Leu Thr Glu Pro Ala Asp Thr Ile Thr Asp Ala Val Lys Val Val Glu Leu Pro Thr

130 140
 Gln Glu Pro Glu Val Gly Ser Thr Cys Leu Ala Ser Gly Trp Gly Ser Ile Glu Pro Glu

150 160
 Asn Phe Ser Phe Pro Asp Asp Leu Gln Cys Val Asp Leu Lys Ile Leu Pro Asn Asp Glu

170 180
 Cys Lys Lys Ala His Val Gln Lys Val Thr Asp Phe Met Leu Cys Val Gly His Leu Glu

190 200
 Gly Gly Lys Asp Thr Cys Val Gly Asp Ser Gly Gly Pro Leu Met Cys Asp Gly Val Leu

210 220
 Gln Gly Val Thr Ser Trp Gly Tyr Val Pro Cys Gly Thr Pro Asn Lys Pro Ser Val Ala

230 238
 Val Arg Val Leu Ser Tyr Val Lys Trp Ile Glu Asp Thr Ile Ala Glu Asn Ser

wherein n is 0 or 1.

5 The kallikrein polypeptides are characterized as the product of procaryotic or eucaryotic host expression (e.g., by bacterial, yeast, Bacillus and mammalian cells in culture) of exogenous DNA obtained by genomic, cDNA or by gene synthesis.

10 The DNA of the present invention includes DNA useful in securing expression in an appropriate host cell of a polypeptide product having the primary structural conformation of a kallikrein polypeptide having an amino acid sequence represented by formula
15 (I) above and one or more of the biological properties of naturally occurring kallikrein. The DNA of the invention are specifically seen to comprise DNA encoding the sequence of formula (I) or their complementary strands. Specifically
20 comprehended are manufactured DNA encoding kallikrein wherein such DNA may incorporate codons facilitating translation of messenger RNA in microbial hosts. Such manufactured DNA may readily be constructed according to the methods of Alton et
25 al., PCT application WO 83/04053.

Also comprehended by the invention are pharmaceutical compositions comprising therapeutically effective amounts of the kallikrein
30 polypeptide products of the invention together with suitable diluents, excipients and/or carriers useful in vasodilation and male infertility applications, etc.

35 The present invention also encompasses the various cloned genes, replicable cloning vehicles, expression vehicles and transformed cultures, all harboring the genetic information necessary to

affect the production of recombinant derived kallikrein polypeptides of the present invention.

Brief Description of the Drawings

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Figure 1 illustrates the construction of pDSHK1.

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Figure 2 is a photograph of a SDS-polyacrylamide gel comparing recombinant human mature kallikrein and naturally-occurring urinary kallikrein wherein the molecular weight markers are designated on the right-hand side and columns 1 and 2 designate urinary kallikrein and recombinant mature kallikrein under nonreducing conditions respectively, and

15

columns 3 and 4 designate urinary kallikrein and recombinant mature kallikrein under reducing conditions.

20

Figure 3 illustrates the construction of pDGHK-L1A.

Detailed Description of the Invention

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According to the present invention, DNA sequences encoding the polypeptide sequence of human species glandular kallikrein of the present invention have been isolated and characterized. Further, the human DNA may be utilized in the eucaryotic and procaryotic expression providing isolatable quantities of polypeptides having biological and immunological properties of naturally-occurring kallikrein as well as in vivo and in vitro biological activities, in particular therapeutic activity, of naturally-occurring kallikrein.

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The DNA of human species origin was isolated from a human genomic DNA library. The isolation of clones containing kallikrein encoding DNA was accomplished

through DNA/DNA plaque hybridization employing a pool of mixed oligonucleotide probes.

5:

The human kallikrein gene of the present invention encodes a 262-amino acid kallikrein polypeptide: a presumptive 17-amino acid signal peptide, a 7-amino acid proenzyme fragment and a 238-amino acid mature protein.

10

Procaryotic or eucaryotic host expression (e.g., by bacterial, yeast and mammalian cells in culture) of exogenous DNA of the present invention obtained by genomic or cDNA cloning or by gene synthesis yields the recombinant human kallikrein polypeptides described herein. The kallikrein polypeptide products of microbial expression in vertebrate (e.g., mammalian and avian) cells may be further characterized by freedom from association with human proteins or other contaminants which may be associated with kallikrein in its natural mammalian cellular environment or in extracellular fluids such as plasma or urine. The products of typical yeast (e.g., Saccharomyces cerevisiae) or procaryote (e.g., E. coli) host cells are free of association with any mammalian proteins. Depending upon the host employed, polypeptides of the invention may be glycosylated with mammalian or other eucaryotic carbohydrates or may be nonglycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue (at position -1).

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Illustrative of the present invention are cloned DNA sequences of monkey and human species origins and

polypeptides suitably deduced therefrom which represent, respectively, the primary structural conformation of kallikrein of monkey and human species origins having the amino acid sequences represented by Table VIII.

The microbially expressed kallikrein polypeptides of the present invention may be isolated and purified by conventional means including, e.g., chromatographic separations or immunological separations involving monoclonal and/or polyclonal antibody preparations, or using inhibitors or substrates of serine proteases for affinity chromatography. Polypeptide products of the invention may be "labeled" by covalent association with a detectable marker substance (e.g., radiolabels, e.g., I^{125} or nonisotopic labels, e.g., biotin) to provide reagents useful in detection and quantification of kallikrein in solid tissue and fluid samples such as blood or urine. DNA products of the invention may also be labeled with detectable markers (for example, radiolabels such as I^{125} or P^{32} and nonisotopic labels such as biotin) and employed in DNA hybridization processes to locate the kallikrein gene position and/or the position of any related gene family in the human, monkey and other mammalian species chromosomal map. The labeled DNA may also be used for identifying the kallikrein gene disorders at the DNA level and used as gene markers for identifying neighboring genes and their disorders.

The kallikrein polypeptide products provided by the present invention are products having a primary structural conformation of a naturally-occurring

5 kallikrein to allow possession of one or more of the biological properties thereof and having an average carbohydrate composition which may differ from that of naturally-occurring kallikrein.

10 Methods for administration of kallikrein polypeptide products of the invention include oral administration and parenteral (e.g., IV, IM, SC, or IP) administration and the compositions of the present invention thus administered would ordinarily include therapeutically effective amounts of product in combination with acceptable diluents, excipients or carriers. Therapeutically effective dosages are
15 expected to vary substantially depending upon the condition treated and may be in the range of 0.1 to 100 µg/kg body weight of the active material. The kallikrein polypeptides and compositions of the present invention may also be lyophilized or made
20 into tablets. Standard diluents such as human serum albumin are contemplated for pharmaceutical compositions of the invention, as are standard carriers such as saline.

25 The kallikrein products of the present invention may be useful, alone or in combination with other factors or drugs having utility in vasodilation and male infertility applications.

* * *

30 The following examples are presented by way of illustration of the invention and are specifically directed to procedures carried out prior to identification of kallikrein encoding monkey cDNA clones and human genomic clones, to procedure
35 resulting in such identification, and to the

sequencing, development of expression systems and immunological verification of kallikrein expression in such systems.

5

Numerous aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following detailed description which provides illustrations of the practice of the invention in its presently preferred embodiments.

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As used herein in the following Examples, unless otherwise specified, the term recombinant kallikrein refers to recombinant mature kallikrein represented by the amino acid sequence of formula (I) wherein n is 0.

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Example 1Protein Sequence of Human Urinary Kallikrein

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A. Amino Acid Sequencing of Human Urinary
Kallikrein and Peptide Fragments

Human urinary kallikrein was purified from
pooled urine of about 30 normal male Caucasian
10 individuals according to the procedures described by
J. Chao et al. [J. Clinical Endocrinology &
Metabolism 51: 840-848 (1980)].

The pure human urinary kallikrein thus obtained was
subject to N-terminus sequence analysis and the
15 first 40 amino acids were identified. The purified
urinary kallikrein protein was derivatized upon
oxidation with performic acid and by reduced
alkylation with dithiothreitol and iodoacetate. The
protein derivatives were then digested using
20 trypsin, Staphylococcus aureus SV-8 protease, and
endolysine-C peptidase. Twenty-nine discrete
peptide fragments were isolated from the digestions.

The peptide fragments thus derived from reduced and
25 alkylated human urinary kallikrein were arbitrarily
assigned numbers according to the protease used
(i.e., T designates peptide fragments derived from
trypsin digestion; S designates peptide fragments
derived from SV-8 protease; and LC designates
30 peptide fragments derived from endolysine C
peptidase). The fragments were analyzed by
microsequence analysis using a gas phase sequencer
(Applied Biosystems), and the amino acid sequence of
the human urinary kallikrein was determined and is
35 represented in Table I. In addition the peptide
fragments obtained from the above digests are also

represented in Table I. In Table I, single letter
designations are employed to represent the deduced
translated polypeptide sequence of urinary
5 kallikrein, an asterisk (*) designates unassigned
amino acids, "NT" designates N-terminal sequencing
of intact protein, "#" designates determined Asn-
glycosylation site and "+" designates unassigned
Asn-glycosylation site.

10 According to the isolation and sequence analysis of
two overlapping peptides, S-18 and LC-17,
represented in Table I, the amino acid at position
162 of human urinary kallikrein protein sequence was
15 identified as lysine.

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Table I
Amino Acid Sequence of Urinary Kallikrein

```

1      10      20      30
I V G G W E C E Q H S O P W O A A L Y H F S T F Q C G G I L
-----
<--S-38-->
<--S-41-->
<-----LC-64----->
40      50      60
V H R Q W V L T A A H C I S D N Y Q L W L G R H N L F D D E
-----
<-----T-50----->
<-----LC-64----->
70      80      90
N T A Q F V H V S E S F P H P G F N M S L L E N H T R Q A D
-----
<--OX-T-43--> + ----- + ----->
<--S-37-->
<-----LC-64-----> + ----- + ----->
100      110      120
E D Y S H D L M L L R L T E P A D T I T D A V K V V E L P T
-----
<--S-7--> <--OX-T-33a--> <--S-22-->
<-----S-61----->
<-----LC-64----->
<-----LC-64-CBb----->
130      140      150
Q E P E V G S T C L A S G W G S I E P E N F S F P D D L Q C
-----
<--T-62, T-58--> # -----
<-----S-52----->
<-----LC-54a, LC-51-----> # -----
160      170      180
V D L K I L P N D E C K K A H V O K V T D F M L C V G H L E
-----
<--OX-T-41, T-52a-->
<-----S-18----->
<-----LC-17-----> <-----LC-45----->
190      200      210
G G K D T C V G D S G G P L M C D G V L Q G V T S W G Y V P
-----
<-----T-53----->
<-----T-57----->
<-----LC-50----->
<-----LC-54b----->
220      230
C G T P N K P S V A V R V L S Y V K W I E D T I A E N S
-----
<--T-29--> <--T-40-->
<--S-45-->
<-----LC-42-----> <-----LC-33----->

```

This result is consistent with the amino acid sequence deduced from human genomic kallikrein DNA sequence as set forth in Table V. This result, however, is different from the reported sequence derived from human pancreatic cDNA and kidney cDNA, wherein glutamic acid was present at position 162 of the reported mature kallikrein, [Baker et al., DNA 4, 445-450 (1985) and Fukushima et al., Biochemistry 24, 8037-8043 (1985)].

B. Glycosylation Sites

It was determined that human urinary kallikrein contains approximately 30% carbohydrate content based on the molecular weight estimated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. The amino acid sequence of human urinary kallikrein indicates that there are three potential Asn-linked glycosylation sites. Sequence analysis of peptide fragments obtained in Example IA., indicates that there are three Asn-linked glycosylation sites (Table II). However, Asn 141 was found only partially glycosylated (60%). This is based on the sequencing results of two peptides containing identical sequence (T-58 & LC-51 vs. T-62 & LC-54A). Asn 141 when linked to carbohydrate, as in fragments T-62 and LC-54a, could not be identified by sequence analysis of the glycopeptide.

Table II
Isolation and Characterization of Glycopeptides

	Fragments No.	Position	Asn-linked	Glycosylation site
5	LC-64	1-114	yes	Asn 78; Asn 84
	T-58 LC-51	115-154	yes	Asn 141
10	T-62 LC-54a	115-154	no	no

Example 2

Design and Construction of Oligonucleotide Probe Mixtures

The amino acid sequence set out in Table I was reviewed in the context of the degeneracy of the genetic codons for the purpose of ascertaining whether mixed probe procedures could be applied to DNA/DNA hybridization procedures on cDNA and/or genomic DNA libraries.

N-terminus amino acid residues 1-10 and 12-29 and Phe-Asp-Asp-Glu-Asn-Thr-Ala-Gln-Phe-Val fragment from a tryptic peptide fragment OX-T-43 (see Table I) were chosen for synthesis of deoxyoligonucleotide probe mixtures and the probe mixtures represented in Table III were designed:

Table III
Oligonucleotide Probes

Probe	Sequence																	
	1	2	3	4	5	6	7	8	9	10								
	Ile	Val	Gly	Gly	Trp	Glu	Cys	Glu	Gln	His								
HK-1a 5'	ATT	GTG	GGC	GGA	TGG	GAA	TGC	GAA	CAA	CA 3'								
				G		G	T	G	G									
HK-1b 5'	ATT	GTG	GGC	GGC	TGG	GAA	TGC	GAA	CAA	CA 3'								
				T		G	T	G	G									
	12					20				29								
	GLN	PRO	TRP	GLN	ALA	ALA	LEU	TYR	HIS	PHE	SER	THR	PHE	GLN	CYS	GLY	GLY	ILE
HK-6a 3'	GTT	GGI	ACC	GTT	CGT	CGG	GAC	ATG	GTG	AAG	AGI	TGI	AAG	GTC	ACA	CCI	CCI	TA 5'
	C			C				A	A	A								
HK-6b 3'	GTT	GGI	ACC	GTT	CGA	CGG	GAC	ATG	GTG	AAG	AGI	TGI	AAG	GTC	ACA	CCI	CCI	TA 5'
	C			C				A	A	A								
HK-7a 3'	GTT	GGI	ACC	GTT	CGA	CGG	GAC	ATG	GTG	AAG	TCG	TGI	AAG	GTC	ACA	CCI	CCI	TA 5'
	C			C				A	A	A								
HK-7b 3'	GTT	GGI	ACC	GTT	CGT	CGG	GAC	ATG	GTG	AAG	TCG	TGI	AAG	GTC	ACA	CCI	CCI	TA 5'
	C			C				A	A	A								
	(I = Inosine)																	
	PHE	ASP	ASP	GLU	ASN	THR	ALA	GLN	PHE	VAL								
KF-2b 5'	TTC	GAC	GAC	GAA	AAC	ACT	GCC	CAG	TTT	GT 3'								
	T	T	T	G		T												

15

The probe mixtures HK-1a and 1b are mixtures of 64 probes 29 nucleotides in length and HK-6a, 6b, 7a and 7b are mixtures of 32 probes, 53 nucleotides in length and probe KF-2b is a mixture of 32 probes, 29 nucleotides in length.

The oligonucleotide probes were labeled at the 5' end with γ - ^{32}P -ATP, 7500-8000 Ci/mmol (ICN) using T_4 polynucleotide kinase (NEN).

Example 3

Hybridization

Probes HK-1a, 1b, 6a, 6b, 7a and 7b were used to hybridize human DNA Southern blots or monkey kidney poly(A)⁺ mRNA blot to determine specific hybridization and for defining the hybridization conditions. Probe mixtures HK-1b at 45°C and HK-7b at 46°C yielded specific hybridization in a hybridization buffer comprising 0.025 pmol/ml of each of the probe sequences in 0.9 M NaCl/5 mM EDTA/50 mM sodium phosphate, pH 6.5/0.5% sarkosyl/100 μg of yeast tRNA per ml. As a result, these two sets of probe mixtures were employed in the monkey kidney cDNA library screening described in Example 4C.

Example 4

A. Monkey cDNA Library Construction

A monkey kidney cDNA library was constructed from poly(A)⁺ mRNA isolated from anemic cynomolgus monkey kidneys as described in PCT Patent Application No. WO 85/02610 and Lin et al., [Gene 44: 201-209, (1986)]. Messenger RNA was isolated from anemic monkey kidneys by the guanidinium

thiocyanate procedure of Chirgwin et al.,
[Biochemistry 18: 5294-5299 (1979)] and poly(A)⁺
mRNA was purified by two runs of oligo(dT)-cellulose
5 column chromatography as described by Maniatis et
al., ["Molecular Cloning, A Laboratory Manual", p.
197-198 Cold Springs Harbor Laboratory, Cold Springs
Harbor, NY, (1982)]. The cDNA library was
constructed according to a modification of the
10 general procedures of Okayama et al. [Mol. and Cell
Biol. 2, 161-170 (1982)]. The procedures are
summarized as follows: (1) pUC8 was used as the
sole vector, cut with PstI and then tailed with
oligo dT of 60-80 bases in length; (2) HincII
15 digestion was used to remove the oligo dT tail from
one of the vector; (3) first strand synthesis and
oligo dG tailing was carried out according to the
Okayama procedure; (4) BamHI digestion was employed
to remove the oligo dG tail from one end of the
20 vector; and (5) replacement of the RNA strand by DNA
was in the presence of two linkers
(GATCTAAAGACCGTCCCCCCCCC and ACGGTCTTTA) in a three-
fold molar excess over the oligo dG tailed vector.

25 B. Bacterial Transformation

Transformation of DNA into E. coli strain DH1
was performed and transformants were selected on LM-
Ap agar containing 1% (w/v) Bacto tryptone/0.5%
(w/v) yeast extract - 10 mM NaCl-10 mM MgSO₄, 1.5%
30 (w/v) Bacto agar containing 50 µg ampicillin per
ml. [Hanahan, J. Mol. Biol. 166: 557-580 (1983)].
Transformants were obtained at a level of 1.5×10^5
per µg of poly(A)⁺ RNA.

C. Colony Hybridization Procedures for Screening
Monkey cDNA Library

5 The colony hybridization procedures employed for
screening the monkey cDNA library were essentially
the same as described in PCT application No. WO
85/02610 and Lin et al. [Gene 44: 201-209, (1986)].

10 Transformed E. coli were spread out at a density of
9000 colonies per 10 x 10 cm plate on nutrient
plates containing 50 µg of ampicillin per ml. Gene
Screen filters (New England Nuclear Catalog No.
NEF-972) were pre-wet on a BHI-CAM plate (Bacto
15 brain heart infusion 37 g/L, casamino acids 2 g/L
and agar 15 g/L, containing 500 µg of
chloramphenicol/ml) and were used to lift the
colonies off the plate. The colonies were grown in
the above medium for 12 hours or longer to amplify
the plasmid copy numbers. The amplified colonies
20 (colony side up) were treated by serially placing
the filters over 2 pieces of Whatman 3 MM paper
saturated with each of the following solutions:

- (1) 50 mM glucose - 25 mM Tris-HCl (pH 8.0) -
10 mM EDTA (pH 8.0) for five minutes;
- 25 (2) 0.5 M NaOH for ten minutes; and
- (3) 1.0 M Tris-HCl (pH 7.5) for three minutes

The filters were air-dried in a vacuum oven at 80°C
for two hours and then treated with a solution
30 containing 50 µg of proteinase K per ml. in Buffer
K, which contains 0.1 M Tris-HCl (pH 8.0), 0.1 M
NaCl, 10 mM EDTA (pH 8.2) and 0.2% sarkosyl.
Specifically, 5 ml of the proteinase K solution was
added to each filter and the digestion was allowed
35 to proceed at 55°C for 30 minutes, after which the
solution was removed.

The filters were treated with 4 ml of a prehybridization buffer [5 x SSPE (0.1M NaCl/5mM EDTA/ 50mM Na Phosphate, pH 6.5) - 0.5% Sarkosyl - 100 µg/ml single stranded E. coli DNA - 5 x BFP (1 x BFP = 0.02% wt./vol. of BSA, Ficoll (M.W. 400,000) and Polyvinylpyrrolidone)]. The prehybridization treatment was carried out at 55°C, generally for 4 hours or longer, after which time the prehybridization buffer was removed.

The hybridization process was conducted as follows: To each filter was added 3 ml of hybridization buffer (5 x SSPE - 0.5% sarkosyl - 100 µg of yeast tRNA per ml) containing 0.075 picomoles of each of the 64 probe sequences of Table III. (the total mixture being designated a HK-1b) and the filters were maintained at 45°C for 20 hours.

Following hybridization, the filters were washed three times for ten minutes on a shaker with 6 x SSC (1 x SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7) - 0.5% sarkosyl at room temperature and washed two to three times with 6 x SSC - 1% sarkosyl at the hybridization temperature (45°C), then autoradiographed. The filters were incubated at 100°C in 1 x SSC, pH 7.0/0.1% sarkosyl for 2 min. to remove the hybridized probes. The filters were again prehybridized as described above and then hybridized with the HK-7b mixed probes at 46°C and washed as described above.

Four positive clones that were hybridized to both HK-7b and HK-1b probe mixtures were obtained among the 200,000 colonies screened and were further confirmed by hybridization to another set of probe mixture KF-2b at 48°C.

One of the positive clones designated as MKK80a was further analyzed and sequenced by the dideoxy method of Sanger et al., [Proc. Natl. Sci. Acad., USA, 74: 5463-5467 (1977)]. The nucleotide sequence of MKK80a clone insert is depicted in Table IV (wherein the arrow "+" designates the beginning of the monkey kallikrein sequence), which is 95% homologous to that of the coding region of the human genomic kallikrein clone λ HK65a as shown in Table V. The amino acid sequence deduced from the nucleotide sequence of the monkey clone MKK80a exhibited 93% homology to that of human kallikrein as illustrated in Table VIII.

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Table IV

MKK80a Clone Nucleotide Sequence

```

      10      20      30      40      50      60
      *      *      *      *      *      *
GA ATT CCC GGG GAT CTT AAA GAC CGT CCC CCC CCC AGC TCC TCC ACC TGC CCG CCC CTG

      70      80      90      100     110     120
      *      *      *      *      *      *
GAC ACC TCT GTC ATC ATG TGG TTC CTG GTT CTG TGC CTC GCC CTG TCC CTG GGG GGG ACT
      Met Trp Phe Leu Val Leu Cys Leu Ala Leu Ser Leu Gly Gly Thr

     130     140     150     160     170     180
      *      *      *      *      *      *
GGT CGT GCG CCC CCG ATT CAG TCC CCG ATT GTG GGA GGC TGG GAG TGT TCC CAG CCC TGG
      Gly Arg Ala Pro Pro Ile Gln Ser Arg Ile Val Gly Gly Trp Glu Cys Ser Gln Pro Trp

     190     200     210     220     230     240
      *      *      *      *      *      *
CAG GCG GCT CTG TAC CAT TTC AGC ACT TTC CAG TGT GGG GGC ATC CTG GTG CAT CCC CAG
      Gln Ala Ala Leu Tyr His Phe Ser Thr Phe Gln Cys Gly Gly Ile Leu Val His Pro Gln

     250     260     270     280     290     300
      *      *      *      *      *      *
TGG GTG CTC ACA GCT GCC CAT TGC ATC AGC GAC AAT TAC CAG CTC TGG CTG GGT CGC CAC
      Trp Val Leu Thr Ala Ala His Cys Ile Ser Asp Asn Tyr Gln Leu Trp Leu Gly Arg His

     310     320     330     340     350     360
      *      *      *      *      *      *
AAC TTG TTT GAT GAC GAA GAC ACA GCC CAG TTT GTT CAT GTC AGT GAG AGC TTC CCA CAC
      Asn Leu Phe Asp Asp Glu Asp Thr Ala Gln Phe Val His Val Ser Glu Ser Phe Pro His

     370     380     390     400     410     420
      *      *      *      *      *      *
CCT GGC TTC AAC ATG AGC CTC CTG AAG AAC CAC ACC CCG CAA GCA GAT GAT TAC AGC CAC
      Pro Gly Phe Asn Met Ser Leu Leu Lys Asn His Thr Arg Gln Ala Asp Asp Tyr Ser His

     430     440     450     460     470     480
      *      *      *      *      *      *
GAC CTC ATG CTG CTC CCG CTG ACG CAG CCT GCC GAG ATC ACA GAC GCT GTG CAG GTC GTG
      Asp Leu Met Leu Leu Arg Leu Thr Gln Pro Ala Glu Ile Thr Asp Ala Val Gln Val Val

     490     500     510     520     530     540
      *      *      *      *      *      *
GAG TTG CCC ACC CAG GAA CCC GAA GTC GGG AGC ACC TGT TTG GCC TCC GGC TGG GGC AGC
      Glu Leu Pro Thr Gln Glu Pro Glu Val Gly Ser Thr Cys Leu Ala Ser Gly Trp Gly Ser

     550     560     570     580     590     600
      *      *      *      *      *      *
ATC GAA CCA GAG AAT TTC TCA TTT CCA GAT GAT CTC CAG TGT GTA GAC CTC GAA ATC CTG
      Ile Glu Pro Glu Asn Phe Ser Phe Pro Asp Asp Leu Gln Cys Val Asp Leu Glu Ile Leu

     610     620     630     640     650     660
      *      *      *      *      *      *
CCC AAT GAT GAG TGC GCC AAA GCC CAT ACC CAG AAG GTG ACA GAG TTC ATG CTG TGT GCC
      Pro Asn Asp Glu Cys Ala Lys Ala His Thr Gln Lys Val Thr Glu Phe Met Leu Cys Ala

     670     680     690     700     710     720
      *      *      *      *      *      *
GGA CAC CTG GAA GGT GGC AAA GAC ACC TGT GTG GGT GAT TCA GGG GGC CCG CTG ACG TGT
      Gly His Leu Glu Gly Gly Lys Asp Thr Cys Val Gly Asp Ser Gly Gly Pro Leu Thr Cys

     730     740     750     760     770     780
      *      *      *      *      *      *
GAT GGT GTG CTC CAA GGT GTC ACA TCA TGG GGC TAC ATC CCT TGT GGC AGC CCC AAT AAG
      Asp Gly Val Leu Gln Gly Val Thr Ser Trp Gly Tyr Ile Pro Cys Gly Ser Pro Asn Lys

     790     800     810     820     830     840
      *      *      *      *      *      *
CCT GCT GTC TTC GTC AAA GTG CTG TCA TAT GTG AAG TGG ATC GAG GAC ACC ATA GCG GAG
      Pro Ala Val Phe Val Lys Val Leu Ser Tyr Val Lys Trp Ile Glu Asp Thr Ile Ala Glu

     850     860     870     880     890     900
      *      *      *      *      *      *
AAC TCC TGA ATG CCC AGC CCC GTC CCC TAC CCC CAG TAA AAT CGA ATG TGC ATC AAA AAA
      Asn Ser —————

     910     920
      *      *
AAA AAA AAA AAA AAA AAA AA

```

Example 5Phage Plaque Hybridization Procedures for Isolating
5 Kallikrein Gene From the Human Genomic Library

A Ch4A phage-borne human fetal liver genomic library prepared according to the procedures described by Lawn et al., [Cell 13: 533-543 (1979)] was obtained and used in a plaque hybridization assay. The phage
10 plaque hybridization procedures employed were as described in PCT No. WO 85/02610 and Lin et al., [Proc. Natl. Sci. Acad., USA 82: 7580-7584 (1985)]. Phage plaques were amplified according to the procedures of Woo, [Methods Enzymol. 68, 389-395
15 (1979)], except that Gene Screen Plus filters and NZYAM plates [NaCl, 5 g; MgCl₂.6H₂O, 2 g; NZ-Amine A, 10 g; yeast extract, 5 g; casamino acids, 2 g; maltose, 2 g; and agar, 15 g (per liter)] were utilized. Phage particles were disrupted by alkali
20 treatment and the DNA's were fixed onto filters (50,000 phage plaques per 8.4 x 8.4 cm filter). The air-dried filters were baked at 80°C for 1 hour and then subjected to proteinase K digestion as described in Example 4. Prehybridization with a 1 M
25 NaCl/1% sarkosyl solution was carried out at 55°C for 4 hours or longer.

The monkey kallikrein MMK80a clone DNA was nick-translated with ³²P-labeled-αdCTP, and the cDNA
30 insert (~1000 bp) was cut out by double-digestions with EcoRI plus HindIII and used as a probe to screen human fetal liver genomic library.

The hybridization buffer contained 2 x10⁵ cpm/ml of
35 the nick-translated monkey kallikrein cDNA in 0.9 M NaCl/5 mM EDTA/50 mM sodium phosphate, pH 6.5/0.5% sarkosyl/100 μg of yeast tRNA per ml. Hybridization

was carried out at 55°C for 20 hours. At the completion of hybridization, the filters were washed three times with 6 x SSC, pH 7.0/0.5% sarkosyl at room temperature and two times at the hybridization temperature, 10 min. per wash.

Two strongly positive clones, designated as λ HK65a and λ HK76a, were obtained among a total of 1.87×10^6 phage plaques screened.

Both human genomic kallikrein lambda clones were subcloned into pUC118 or pUC119 and the double-stranded DNA's were sequenced according to the procedure of Chen et al., [DNA 4, 165-170 (1985)] using the dideoxy method of Sanger et al., [Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977)], and the nucleotide sequence of kallikrein gene containing region for clone λ HK65a is represented in Table V.

20

25

30

35

Clone λ HK65a Genomic Sequence

[illegible]

Table V (Continued)

[illegible]

Example 6

5 Table VI represents the nucleotide sequence of a
manufactured DNA encoding the recombinant mature
kallikrein polypeptide of the present invention.
The manufactured DNA was constructed according to
the methods described by Alton et al., PCT
application WO 83/04053. This manufactured
10 (synthetic) gene has codons preferred for E. coli
expression. EcoRI and NdeI sites were added 5' to
the initiation codon ATG, and PstI and BamHI sites
3' to the termination codon TAA. The resulting
sequence is depicted in Table VI-A. The entire
15 synthetic gene was cloned into pUC119 cut with EcoRI
and BamHI and the resulting plasmid then
sequenced. The gene was removed by digestion with
NdeI and BamHI, then inserted into the E. coli
expression vector pCFM1156 as described in commonly
owned U.S. Ser. No. 004,379 hereby incorporated by
20 reference. The resulting expression plasmid was
used to transfect E. coli host FM5 as described in
Burnette et al., BIO/TECHNOLOGY, Vol. 6, 699
(1988). The transformed E. coli was grown in
25 brain/heart infusion (BHI) medium containing 20
µg/ml kanamycin at 28°C until OD₆₀₀ = 0.1, then
shifted to 42°C for 4-6 hr for maximal expression.
The level of kallikrein expression was approximately
25% of the total cellular protein as estimated from
30 SDS - Polyacrylamide gel electrophoresis analysis of
whole cell lysates.

The E. coli expressed kallikrein was extracted from
inclusion bodies isolated from a bacterial cell
35 paste by solubilizing in 8 M urea, pH 3.5 for 2
hr. The lysate was clarified by centrifugation at

5000 x g for 30 minutes. The clear lysate was diluted 10-fold and adjusted to pH 9-11 with sodium hydroxide. The solution was left stirring overnight at 4°C, then 2-mercaptoethanol added to a final concentration of 0, 50, or 100 mM. At the completion of oxidation, pH of the solution was adjusted to 8 with acetic acid and then reclarified as before.

The efficiency of refolding into an immunologically detectable molecule was determined by RIA as described in Example 7 to be approximately 0.42% of the proteins present in inclusion bodies.

												30	NcoI								60
ATG	ATT	GTA	GGC	GGT	TGC	GAA	TGT	GAA	CAA	CAT	AGC	CAG	CCA	TGG	CAG	GCT	GCG	CTG	TAT		
Met	Ile	Val	Gly	Gly	Trp	Glu	Cys	Glu	Gln	His	Ser	Gln	Pro	Trp	Gln	Ala	Ala	Leu	Tyr		
												90									120
CAC	TTT	TCT	ACC	TTT	CAA	TGC	GGC	GGT	ATC	CTG	GTG	CAC	CGT	CAG	TGG	GTT	CTG	ACC	GCG		
His	Phe	Ser	Thr	Phe	Gln	Cys	Gly	Gly	Ile	Leu	Val	His	Arg	Gln	Trp	Val	Leu	Thr	Ala		
												150									180
GCA	CAC	TGC	ATC	AGC	GAT	AAT	TAT	CAA	CTG	TGG	CTC	GGC	CGC	CAC	AAC	CTG	TTC	GAT	GAC		
Ala	His	Cys	Ile	Ser	Asp	Asn	Tyr	Gln	Leu	Trp	Leu	Gly	Arg	His	Asn	Leu	Phe	Asp	Asp		
												210									240
GAA	AAC	ACT	GCA	CAG	TTC	GTT	CAC	GTG	AGC	GAA	TCC	TTT	CCG	CAC	CCG	GGC	TTC	AAC	ATG		
Glu	Asn	Thr	Ala	Gln	Phe	Val	His	Val	Ser	Glu	Ser	Phe	Pro	His	Pro	Gly	Phe	Asn	Met		
												270	XhoT								300
TCT	CTG	CTC	GAG	AAT	CAC	ACC	CGT	CAG	GCG	GAT	GAA	GAC	TAT	AGC	CAT	GAC	CTG	ATG	CTG		
Ser	Leu	Leu	Glu	Asn	His	Thr	Arg	Gln	Ala	Asp	Glu	Asp	Tyr	Ser	His	Asp	Leu	Met	Leu		
												330									360
CTG	CGT	CTG	ACC	GAA	CCG	GCA	GAT	ACC	ATC	ACC	GAT	GCG	GTT	AAA	GTG	GTT	GAA	CTG	CCG		
Leu	Arg	Leu	Thr	Glu	Pro	Ala	Asp	Thr	Ile	Thr	Asp	Ala	Val	Lys	Val	Val	Glu	Leu	Pro		
												390									420
ACT	CAG	GAA	CCG	GAA	GTG	GGC	TCC	ACC	TGT	CTG	GCG	TCT	GGT	TGG	GGC	AGC	ATC	GAA	CCG		
Thr	Gln	Glu	Pro	Glu	Val	Gly	Ser	Thr	Cys	Leu	Ala	Ser	Gly	Trp	Gly	Ser	Ile	Glu	Pro		
												450									480
GAA	AAC	TTC	AGC	TTC	CCG	GAT	GAC	CTG	CAA	TGC	GTG	GAC	CTG	AAA	ATT	CTG	CCG	AAC	GAC		
Glu	Asn	Phe	Ser	Phe	Pro	Asp	Asp	Leu	Gln	Cys	Val	Asp	Leu	Lys	Ile	Leu	Pro	Asn	Asp		
												BstEII								540	
GAA	TGC	GAA	AAA	GCG	CAC	GTG	CAA	AAG	GTT	ACC	GAT	TTC	ATG	CTG	TGC	GTG	GGC	CAT	CTG		
Glu	Cys	Glu	Lys	Ala	His	Val	Gln	Lys	Val	Thr	Asp	Phe	Met	Leu	Cys	Val	Gly	His	Leu		
												570									600
GAG	GST	GGT	AAA	GAT	ACG	TGT	GTG	GGT	GAT	TCT	GGC	GGC	CCG	CTG	ATG	TGC	GAC	GGT	GTT		
Glu	Gly	Gly	Lys	Asp	Thr	Cys	Val	Gly	Asp	Ser	Gly	Gly	Pro	Leu	Met	Cys	Asp	Gly	Val		
												630									660
CTT	CAG	GGC	GTT	ACC	AGC	TGG	GGT	TAC	GTT	CCG	TGT	GGT	ACC	CCG	AAC	AAA	CCG	TCT	GTG		
Leu	Gln	Gly	Val	Thr	Ser	Trp	Gly	Tyr	Val	Pro	Cys	Gly	Thr	Pro	Asn	Lys	Pro	Ser	Val		
												690									720
GCG	GTT	CGT	GTG	CTG	AGC	TAC	GTT	AAA	TGG	ATC	GAA	GAT	ACC	ATT	GCG	GAG	AAC	AGC	TAA		
Ala	Val	Arg	Val	Leu	Ser	Tyr	Val	Lys	Trp	Ile	Glu	Asp	Thr	Ile	Ala	Glu	Asn	Ser	End		

Pst I
CTGCAG
GACGTCCTAG
BamHI

Example 7Radioimmunoassay for Kallikrein

5 The radioimmunoassay procedure employed for quantitative detection of kallikrein was a modification of the procedure described by Shimamoto et al., [J. Clin. Endocrinol & Met. 51: 840-848 (1980)]:

10 The assay buffer employed was phosphate-buffered saline (PBS; 0.14 M NaCl in 0.01 M $\text{Na}_2\text{HOP}_4\text{-NaH}_2\text{PO}_4$, pH 7.0) containing 0.1% BSA. The assay utilizes the following reagents and samples: 200 μl aliquots of
15 samples or purified human urinary kallikrein (24 pg - 6250 pg) standards containing appropriate dilutions in the assay buffer; 100 μl of rabbit antiserum raised against purified human urinary kallikrein at 1:250,000 dilution; 100 μl of ^{125}I -
20 human urinary kallikrein (Sp. Act. $\sim 1.5 \times 10^8$ cpm per μg kallikrein) 50,000 cpm. All samples and standards were assayed in duplicate. Assay tubes were incubated at 37°C for 2 hours then at 4°C overnight. Antibody bound kallikrein was separated
25 from free kallikrein by adding a formalin fixed staphylococcus aureus (Cowan Strain) cell suspension, IgG-sorb (Enzyme Center, Inc). To each tube, fifty μl of 10% IgG-sorb was added and let the reaction mixture stand at room temperature for 30
30 min. The tubes were centrifuged and the resulting pellet was washed twice with a wash buffer comprising 0.05 M Tris-HCl, (pH 8.9), 2% BSA, 0.1% SDS and 0.1% Triton X-100 and counted in a gamma
35 counter. The assay detects kallikrein in a range from about 24 pg to about 6250 pg. The kallikrein content of an unknown sample was determined by

comparison to a standard containing a known quantity of pure human urinary kallikrein.

5

Example 8

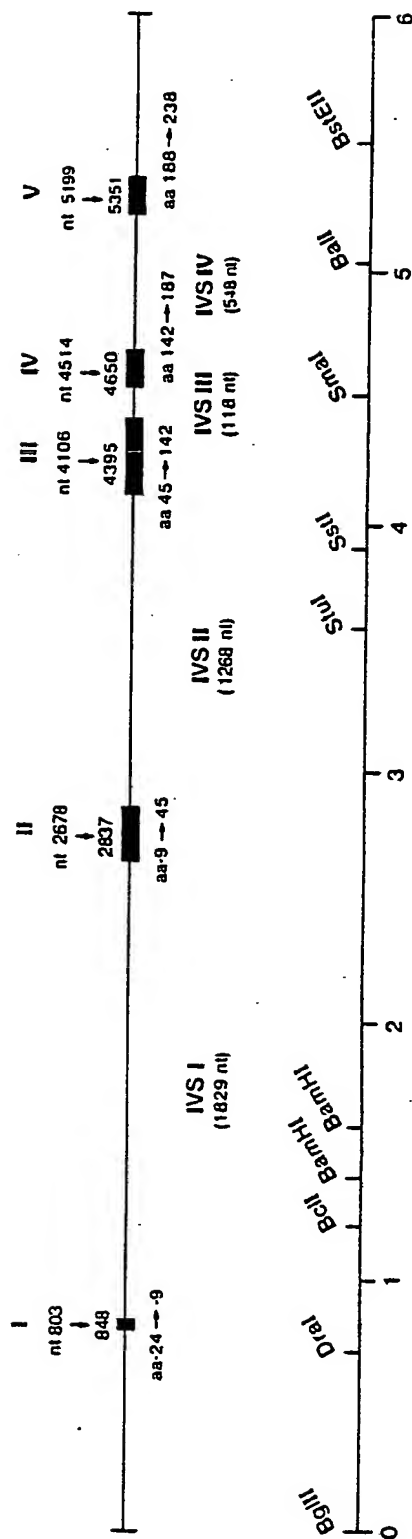
Human Grandular Kallikrein Gene Sequences

10 Nucleotide sequence analyses of the two independent positive human genomic kallikrein clones designated as λ HK65a and λ HK76a were carried out and results obtained for the kallikrein gene containing region of clone λ HK65a are set out in Table V. Both clones
15 have identical kallikrein protein coding sequences and identical intron sequences that were completed and the restriction endonuclease map of the human kallikrein gene from clone λ HK65a is shown in Table VII.

20

The protein coding region of the gene is divided by four intervening sequences or introns. Since the transcription initiation site of the mRNA for kallikrein has not been determined due to lack of
25 human tissue mRNA, the boundary on the 5' side of exon I is undefined. The exons were identified by comparison of the nucleotide sequence to the amino acid sequence of human urinary kallikrein shown in Table I and by comparison with the cDNA sequence
30 published [Baker et al., supra, and Fukushima et al., supra]. The exon-intron boundaries of the kallikrein gene conform to consensus splice rules, [Mount, Nucleic Acids Res. 10: 459-472 (1982)]. In Table IV, the initial sequence appears to comprise
35 the 5' end untranslated region (802 bp) of the gene which may contain enhancer/promoter like functions,

Table VII



that leads up to a translated DNA region, the first exon, coding for the first 15 amino acids (-24 through -10, and part of residue -9). Then follows an intervening sequence (IVS) of 1829 base pairs. The second exon immediately followed codes for amino acid residues glycine -9 through aspartic acid 45. The second IVS that follows comprises 1268 base pairs. The third exon codes for amino acid residues aspartic acid 45 through phenylalanine 142, the third IVS of 118 base pairs. The fourth exon encodes amino acid residues phenylalanine -142 through valine 187, the fourth IVS of 548 base pairs. The last exon codes for amino acid residues Glycine 188 through serine 238 and a stop codon (TGA).

There is a 46 bp untranslated region at the 3' end of the last exon. The nucleotide sequence 15 bp upstream from the site contain a sequence AGTAAA resembling the consensus polyadenylation signal sequence AATAAA and the related sequences normally found at this location. [Nevins, Annu. Rev. Biochem. 52, 441-466 (1983)].

The kallikrein gene encodes a protein of 262 amino acids. Based on the NH₂-terminal amino acid sequence of purified human urinary kallikrein, the last 238 residues correspond to the mature active protein with a calculated M_r of 26403 in an unglycosylated form. The sequence of the first 17 amino acids, predominantly hydrophobic residues, is consistent with this region encoding a signal peptide, [Watson, Nucleic Acids Res. 12: 5145-5164 (1984)] and the following 7 amino acid residues being the activation peptide or propeptide, [Takahashi et al., J. Biochem 99, 989-992 (1986)].

The amino acid sequence starting at position -7 through -1 corresponds to the propeptide

sequence in the recombinant prokallikrein and that
starting at +1 corresponds to the sequence of the
amino terminus of expressed recombinant mature
5 kallikrein product of the present invention in CHO
cells. As indicated in Table V, the mature protein
has three potential sites for Asn-linked
glycosylation, amino acid residues 78, 84 and 141 in
the third exon of the gene, according to the rule of
10 Asn-Xaa-Ser/Thr, [Marshall, Biochem. Soc. Symp. 40,
17-26 (1974)], and has 10 cysteine residues.

Example 9

15 Table VIII below illustrates the extent of
polypeptide sequence homology between human and
monkey kallikrein of the present invention. In the
upper continuous line of the Table, three letter
designations for amino acids are employed to
20 represent the deduced translated polypeptide
sequences of human kallikrein of the present
invention commencing with residue -24 and the amino
acid residues appearing in the lower continuous line
shows the differences in the deduced polypeptide
25 sequence of monkey kallikrein also commencing at
assigned residue number -24. Dashes are employed to
highlight missing amino acid residues in the monkey
kallikrein sequence that are present in human
kallikrein of the present invention.

30

35

Example 10Construction of the Plasmid pDSHK1 for the
Expression of Human Kallikrein Gene in Mammalian
Cells

For the expression of the human kallikrein gene, a 7 kb Bgl II-XbaI fragment from lambda clone λ HK65a, that contains the entire kallikrein gene, was first isolated and then inserted into BamHI/XbaI doubly-digested plasmid vector pUC118. The resulting pUC118-based kallikrein clone was subjected to the Henikoff deletion procedures [Henikoff, Gene 28: 351-359 (1984)] to generate clones containing the human kallikrein gene with its 3'-flanking sequences deleted to various extent. In particular, the plasmid was opened by digesting with restriction enzymes SphI and SalI. The combination of ExoIII nuclease and SI nuclease was utilized to digest the insert from its 3' end (the XbaI site) for various time intervals. Thereafter, the plasmid DNA was treated with DNA polymerase large fragment (Klenow enzyme) to fill the ends for subsequent blunt-end ligation with T₄ DNA ligase. The recircularized plasmid DNA was used to transform the DH5 α E. coli host, (Bethesda Research Laboratories, Cat. No. 8263SA). The resulting transformants were analyzed by sizing the inserts and by DNA sequencing. One of the deletion clones, pHK102, contains the entire human kallikrein gene insert with 801 bp upstream from the protein initiation codon and 232 bp downstream from the termination codon. The pHK102 contains a DraI site 67 bp upstream from the protein initiation codon of the kallikrein gene and a HindIII site which was carried over from the pUC118 237 bp downstream from the termination codon. The pHK102 DNA was digested with DraI and HindIII and

the approximate 4.85 Kb DraI-HindIII fragment was isolated and briefly digested with Bal31-Slow nuclease. The resulting DNA fragment was ligated to the BamHI cleaved expression vector pDSVL (pDSVL contains a murine dihydrofolate reductase gene and SV40 late promoter as described in PCT application No. WO 85/02610), that have been end-filled with DNA polymerase large fragment (Klenow enzyme). The kallikrein expression plasmid thus obtained was designated as pDSHK1, which contains the entire human kallikrein gene including 64 bp 5' to the initiation codon and 232 bp 3' from the termination codon. The detailed construct of pDSHK1 is depicted in Figure 1. The pDSHK1 plasmid contains a murine DHFR minigene as a EcoRI-PstI fragment from pMg1; SV40 origin of replication and early/late promoters in the HindIII-BamHI fragment; SV40 nt 2538-2770 in the BamHI-BclI fragment; pBR322 nt 2448-4362 in the HindIII-EcoRI fragment; and the 4.85 kb DraI-HindIII fragment of the human kallikrein gene. The SV40 late promoter is used to drive the expression of the human kallikrein gene. This pDSHK1 plasmid was used to transfect African green monkey kidney cells COS-1 for transient expression or transfect DHFR⁻ CHO cells for stable expression.

Example 11

A. Transient Expression of Human Kallikrein Gene in African Green Monkey Kidney Cells, COS-1 Cells

A calcium phosphate microprecipitate of covalent circular DNA, pDSHK1, at 1.5 µg DNA plus 10 µg mouse liver DNA as carrier per 4×10^5 cells was used to transfect COS-1 cells. The cells were grown in high glucose DMEM medium supplemented with 10% fetal bovine serum plus penicillium, streptomycin and

glutamine. A three-day conditioned medium was determined to contain 5.1 ng immunoreactive kallikrein per ml using the radioimmunoassay procedure of Example 7.

B. Expression of Human Kallikrein Gene in Chinese Hamster Ovary (CHO) Cells with Plasmid pDSHK1

(1) Transfection of CHO Cells With the Circular Plasmid DNA of pDSHK1

Chinese hamster ovary DHFR⁻ cells (CHO DHFR⁻ cells) [Urlaub et al., (1980) Proc. Nat. Acad. Sci. (U.S.A.), 77, 4461] lack the enzyme dihydrofolate reductase (DHFR) due to mutations in the structural genes and therefore require the presence of glycine, hypoxanthine, and thymidine in the culture media for growth. The cells were grown as a monolayer in medium C comprising Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 1% nonessential amino acids, 1% hypoxanthine and thymidine, and penicillin, streptomycin, and glutamine. One day prior to transfection, cells were plated at the approximate density of 3×10^6 cells per 10 cm culture dish. A calcium phosphate microprecipitate procedure by a modification of the methods of Graham et al., [Virology 52: 456-467 (1973)], and Wigler et al., [Cell 11: 223-232 (1977)] was employed to introduce pDSHK1 DNA with a final concentration of 6 to 7 μ g of plasmid DNA per 10^6 cells. Cells that had been transformed with and were expressing the DHFR gene (and thereby the kallikrein gene) survived and proliferated in the selective medium comprising DMEM supplemented with 10% dialyzed fetal bovine

serum, 1% nonessential amino acids, glutamine, penicillin, and streptomycin, but no hypoxanthine and thymidine. The cells that grew in the selective medium were considered as stable transformants. The culture conditioned medium of the stable transformants was collected and assayed for the immunoreactive human kallikrein by the radioimmunoassay procedure, described in Example 7. The results indicated that the immunologically reactive recombinant human kallikrein products were secreted at the level of 6 ng/ml from a 3-day conditioned medium.

(2) Transfection of CHO cells with linear DNA fragment of pDSHK1

Plasmid pDSHK1 (9.85 kb) was digested by restriction enzymes EcoRI and HindIII. The resulting digest was applied to a 0.7% agarose gel electrophoresis to separate the kallikrein insert containing fragment from the prokaryotic DNA fragment which originated from pBR plasmid. The 7.9 Kb DNA fragment, that contains the DHFR gene, the SV40 regulatory sequences, and the kallikrein gene, was isolated from the agarose gel. The transfection of CHO cells with this linear DNA fragment was carried out in 60 mm culture dishes at the cell density of 1×10^6 per dish with 10 μ g of 7.9 Kb DNA fragment added per dish as previously described in Example 11B(1) for the closed circular DNA. Seventeen hours after adding the DNA to the cells, the medium was aspirated and replaced with a fresh medium. Three days later, the cells were trypsinized from two of 60 mm dishes and transferred to two 75-cm² culture flasks. On

the following day, the medium was replaced with the selective medium. From this point on, the cells were maintained and subcultured in the selective medium for approximately four weeks and were designated as stable transformants. The stable transformants have a secretion level of 0.27 µg/ml of conditioned medium obtained from a 14-day culture in serum-free condition, Ham's F-12/DME 1:1 mixture supplemented with glutamine.

C. Amplification of the Cloned Human Kallikrein Gene (HK) in CHO DHFR⁻ Cells Containing pDSHK1

The quantity of recombinant kallikrein produced by the stable transformants may be increased by gene amplification with methotrexate treatment to yield new cell strains having higher productivity. To amplify the cloned kallikrein gene, the stable transformants were subjected to a series of increasing concentration of methotrexate treatment. Initially, the cells were shifted from the selective medium to a methotrexate medium. The methotrexate medium is composed of DMEM supplemented with 5% fetal calf serum, 1% nonessential amino acids, glutamine, penicillin and streptomycin, plus methotrexate at desired concentration.

Cell strain CHO-DSHK1-C1 (stable transformants that originate from circular DNA transfected cells) was subjected to treatments with increasing methotrexate concentrations (0 nM, 20 nM, 60 nM, 300 nM, and 1 µM) to select out the higher producing cell strains. After about four weeks of culturing in a given methotrexate-containing media, the near confluent cells were then fed with serum-free media without methotrexate. Representative 7-day medium samples from serum-free culture (Ham's F-12/DMEM 1:1

mixture) from each amplification step were assayed using the radioimmunoassay procedure of Example 7 and determined to contain 10, 42, 110, 693 and 708 ng human kallikrein/ml, respectively. On the other hand, the cell strain CHO-DSHK1-L1 (stable transformants that originate from linear DNA transfected CHO cells) was also subjected to amplification with increasing concentrations of methotrexate (0 nM, 30nM, 60 nM, 300 nM, 1 μ M and 5 μ M). The kallikrein content in the conditioned media was determined using the radioimmunoassay (RIA) procedure described in Example 7. The results based on 7-day samples are shown in Table IX.

Table IX

Expression of Kallikrein by CHO-DSHK1-L1 Cells
Different Stages of Amplification with Methotrexate

<u>Methotrexate Concentration</u>	<u>Culture Media</u>	<u>Days of Conditioned Media</u>	<u>Kallikrein Concentration (μg/ml)</u>
0	SF ¹	14	0.27
60 nM	SF	7	1.44
	SF+PC-1 ²	7	1.92
300 nM	SF+PC-1	7	2.34
	SF+PC-1+Gln ³	7	3.57
	SF+PC-1 ⁴	7	4.06
1 μ M	SF+PC-1	7	1.93
		10	2.80
		12	4.87
	5% serum	7	1.74
5 μ M	5% serum	7	0.82

1. SF = serum-free medium, i.e. Ham's F12/DMEM 1:1 mixture.
2. PC-1 = a supplement for serum-free culture, obtained from Ventrex (Portland, Maine).
3. Glutamine concentration is 8 mM instead of the regular 2 mM.
4. The cells used in this experiment had been previously maintained for 7 days in PC-1 supplemented serum-free medium, and subsequently 3 days in methotrexate medium.

The highest expression level was 4.06 μ g human kallikrein/ml for a 7-day conditioned medium,, observed with 300 nM methotrexate treated cells that have been transfected with linearized kallikrein DNA.

D. Expression of Human Kallikrein Gene in CHO cells with Plasmid pDGHK-L1A

(1) Construction of pDGHK-L1A Expression Vector

pDGHK-L1A as depicted in Figure 3, consists of the following: (i) pBR322 nt 2448-4362 as a HindIII-EcoRI fragment; (ii) a DHFR minigene as an EcoRI-PstI fragment from pMgl' with a deletion in the 3' untranslated region made by removing a BglIII-BglIII fragment of 556 bp, and then end filled with klenow enzyme; (iii) A bidirectional SV40 termination sequence of 237 bp originally as a BamHI-BclI fragment (SV40 nt 2770-2553), adding synthetic linker-1 to BclI (destroying the BclI site and generating a PstI site), and adding synthetic linker-2 to BamHI end of the fragment to create a SalI site; (iv) The kallikrein gene (nt 801- nt 5370 in Table V) with synthetic linker-3 added to DraIII site at nt 801 and with a SalI site was created by insertion of GA using site-directed mutagenesis between nt 15 and nt 16 past termination codon TGA, generating the sequence TGAACGCCCAGCCCTGTA(GA)C; (v) A rat glucose-regulated protein (GRP78) gene promoter which contains SmaI-BssHII fragments (GRP78 nt-722 to nt-37) [Shin C. Chang et al., Proc. Natl. Acad. Sci. 84:680-684 (1987)]; and (vi) synthetic linker-4 to reinstate the sequence including the mRNA cap site and also to create a BamHI cloning site. The GRP promoter-kallikrein gene fusion was made by ligating the BamHI site of linker-4 to the

BglIII site of linker-4 to the BglIII site of linker-3. Linker-5 was added to the SmaI end of the GRP promoter sequence and it was joined to the pBR322 derived sequence at HindIII.

5 Linker 1:

GCAATGGCAACAACGTTGCCCG
ACGTCGTTACCGTTGTTGCAACGGGCCTAG
PstI (BclI)

10 Linker 2:

SaII BamHI
TCGACTG
GACCTAG

Linker 3:

BglIII (DraIII)
GATCTCAAACAGACAACAT
AGTTTGTCTGTT

15 Linker 4:

CGCGCTCGATACTGGCTGTGACTACACTGACTTGGACACTTGGCCTTTTGC GG GTTTGAG
GAGCTATGACCGACACTGATGTGACTGAACCTGTGAACGGGAAAACGCCAAACTCCTAG
BssHI BamHI

20 Linker 5:

Hind III EcoRI SmaI
AGCTTGAGTCCTGAATTCGAGCTCGGTACCC
ACTCAGGACTTAAGCTCGAGCCATGGG

25 (2) Transfection of CHO Cells with Plasmid

pDGHK-L1A:

CHO D⁻ cells were grown in high glucose, high glutamine DMEM (Gibco Laboratories, Cat. #320-1965) supplemented with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 13.6 µg/ml hypoxanthine, 7.6 µg/ml thymidine, and 2 mM glutamine. The 60 mm plates were seeded at a density of approximately 3.3×10^5 cells/dish containing 5 ml of the medium and allowed to grow for about 20 hours at 37°C, 5% CO₂. The media from each plate was aspirated, fresh media added and cells allowed to grow an additional five hours at 37°C, 5% CO₂. The

media was again aspirated and the cells washed once with PBS, then once with transfection buffer before cells were transfected with the linearized plasmid DNA, pDGHK-L1A. The transfection procedure was essentially the same as described by Bond and Wold (Bond, V.C. and Wold, B., Mol. & Cell. Biol. 7:2286-2293, 1987). The transfection buffer consisted of 5 mM NaCl, 120 mM KCl, 1.5 mM Na₂HPO₄ and 25 mM Tris, pH 7.5; then adding CaCl₂ to 1.4 mM and MgCl₂ to 0.5 mM. After mixing, the buffer was sterilized by filtering through a 0.22 µm Costar Microstar filter. Plasmid pDGHK-L1A, linearized with restriction enzyme PvuI was extracted once with phenol-chloroform, 1:1, saturated with 0.05 M Tris, 0.02 M EDTA, 0.01 M 2-mercaptoethanol, pH 8.0, then once with chloroform only. The extracted plasmid digest was precipitated with 1/2 volume 7.5 M ammonium acetate and 2 1/2 volumes absolute ethanol at -20°C overnight. Precipitated plasmid DNA was redissolved in the sterile transfection buffer to give a final concentration of approximately 2.5 µg/ml and 5 µg/ml. Poly-L-ornithine (Sigma, Cat. #P4638), 200 mg/ml in 0.01 M Tris pH 7.5 sterilized through a 0.22 µm Costar Microstar filter, was added to 17 µg/ml and a plasmid DNA concentration of 2.5 µg/ml transfection mixture, and was added to 25 µg/ml and a plasmid DNA concentration of 5 µg/ml plasmid DNA transfection mixture. Transfection mixtures (0.5 ml/60 mm dish) were carefully layered onto cells containing dishes and incubated for 1 hour at room temperature in a laminar flow hood. At the end of the transfection treatment, 5 ml of culture media were added to each dish, which was then returned to 37°C, 5% CO₂. The following

day, i.e., approximately 16 hours later, the culture dishes were aspirated and fresh media added, returning the dishes to 37°C, 5% CO₂ for 48 hours. Cells were trypsinized and replated to four 100-mm dishes (10 ml medium each) for each 60 mm dish. One day later, the media was aspirated and the cells were washed twice with PBS. Then the selective media (it contains high glucose, high glutamine DMEM supplemented with 10% dialyzed FBS, 2 mM glutamine, 0.1 mM non-essential amino acids) was added. Selective media was changed approximately every 3 days until 15 days post transfection, when individual colonies were visible. Colonies were tabulated, trypsinized and transferred to 25-cm² culture flasks, at a density of approximately 50 pooled colonies/flask. From 1 µg of transfected DNA, about 40-50 colonies were obtained.

(3) Amplification of the Cloned HK Gene in CHO DHFR-Cells Containing pDGHK-L1A

Amplification procedure for CHO DHFR⁻cells containing linearized pDGHK-L1A was essentially the same as that described in Example 11C with the following modifications.

Cell strain CHO-DGHK-L1A-8 (Stable transformants that originate from pDGHK-L1A transfected cells) was subjected to two different schemes of treatment with methotrexate (MTX). One scheme of MTX concentration was:

0→20nM→60nM→200nM→600nM MTX

and the other was:

0→30nM→100nM→300nM→900nM.

The kallikrein content as secreted by the cells into the media was determined using RIA as described in Example 7. 60nM MTX and 100nM MTX

amplified cells secreted the highest level of kallikrein, 1350ng/ml/day and 1725 ng/ml/day respectively. The results are shown in Table X below.

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TABLE X

The Expression Level of Kallikrein by
CHO-DGHL1A-8 Cells
at Different Stages of Amplification with Methotrexate

	<u>MTX (nM)</u>	<u>Daily Yield of rHK (ng/ml)</u>
10	0	630
	30	561
15	60	1350
	100	1725
	200	191
20	300	61
	600	167

25

Data was obtained from 175-cm² culture flasks containing 40 ml medium and approximately 1.5×10^7 cells per flask. The medium used was DMEM/F12 1:1 containing PC-1 (10ml/500 ml medium), 6mM Glutamine and 0.1 mM non-essential amino acids.

30

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Example 12Isolation of Recombinant Human Kallikrein

5 Cell free culture media containing recombinant human kallikrein produced by CHO cells was pooled and concentrated using a diafiltrator having a 10,000 dalton molecular weight cutoff membrane filter. The concentrated sample was buffer exchanged with 10
10 volumes of 10 mM Tris-HCl buffer (pH 7.8). The crude recombinant kallikrein was added to a QA-sepharose column packed and equilibrated with 10 mM Tris-HCl buffer (pH 7.8). The column was washed with the equilibration buffer and the recombinant
15 kallikrein was eluted with a linear gradient of from 0 to 0.5 M NaCl in the same buffer. Both active kallikrein and enzymatically inactive prokallikrein were eluted at fractions containing 0.3 to 0.4 M NaCl. The recombinant kallikrein and prokallikrein
20 fractions were pooled and dialyzed against 10 mM Tris-HCl (pH 7.8) buffer to remove excessive sodium chloride. The dialysis proceeded for 2-3 days at 4°C, at which time prokallikrein was completely activated by kallikrein to generate mature
25 kallikrein. The dialyzed activated kallikrein pool was added to a benzamidine-sepharose affinity column which was pre-equilibrated with 10 mM Tris-HCl (pH 7.8). The column washed with the equilibration buffer and kallikrein was eluted with the same
30 buffer containing 2 M guanidine HCl. The kallikrein fractions were pooled then precipitated with 70% saturated ammonium sulfate in 10 mM Tris-HCl (pH 7.8). The ammonium sulfate precipitated kallikrein fractions were dissolved in 10 mM Tris-HCl buffer
35 (pH 7.8) and the dissolved sample was applied to a sephacryl S-300 gel filtration column which is equilibrated with the same buffer. The kallikrein fractions were eluted at molecular weights ranging

from 30,000 to 45,000. The pooled kallikrein fractions contained highly purified active recombinant human mature kallikrein. A C₄ HPLC column (Vydac C₄ column 25 cm x 4.6 mm) chromatography was used to check the purity of the final product. The buffer gradient conditions used were as follows:

Gradient:

t(min)	%A	%B
0	75	25
60	30	70
70	30	70
71	75	25

A = 0.1% TFA (Trifluoroacetic acid)

B = 90% CH₃CN/0.1%TFA (CH₃CN = Acetonitrile)

Flow = 1 ml/min

The results are shown below in Table XA:

TABLE XA

Isolation of Recombinant Human Tissue Kallikrein (TCHK009) Derived from CHO Cells

Steps	Volumes (ml)	Protein (OD ₂₈₀)	Enzyme ** Activity (units)	Specific Activity (U/OD)	RIA (mg)	Yield (%)	Purification fold	Comment
Culture Media	16,000	-	4,400	-	(40 mg)	-	-	
Diafiltration	1,500	3,720	4,610	1.24	(40 mg)	100	1	
QA-Sepharose	200	432	6,750	15.63		-	8.6	
Dialysis- Autoactivation	220	432	54,400	125.93	44	110	-	
Benzamidine- Sepharose								
Fr A ***	40	57	20,000	350.87	-	72	33	
Fr B	24	55	18,130	329.64				
Sephacryl S-300								
Fr A	37	27	14385	532.78	15.	69	84	30% proform 70% active form
Fr B	33	17	13407	788.65	11.3			7% proform 93% active

* HUK concentration of 1 mg/ml has an OD = 1.6 at 280 nm.

Specific activity = 1100 U/mg assayed by AcPheArg0et coupling enzyme procedure.

** Kallikrein activity was assayed by protease activity using AcPheArg0et as a substitute (Piedler *et al.*, Methods in Enzymology 80: 493-532, 1980).

*** Frx=Fraction

5 The resulting purified kallikrein was enzymatically active as determined using the following synthetic peptide: Ac-Phe-Arg-OEt-Nitroanilide as a substrate for an esterase assay according to the procedures described by Geiger et al., [Adv. Biosci. 17, 127 (1979)].

10 From different batches of culture media both kallikrein (the mature, active form) and prokallikrein (the inactive form) have been purified using similar purification procedures. The purified proenzyme was not enzymatically active. Active kallikrein was isolated from prokallikrein after
15 autoactivation or after removal of the activation peptide in prokallikrein by incubation with trypsin.

Example 13

20 Sequence Analysis of Recombinant Kallikrein and Prokallikrein

Sequence determination of the purified recombinant kallikrein revealed that the purified protein is
25 homogeneous and contains a single amino terminus. The partial N-terminal sequence is determined as:

1 2 3 4 5 6 7 8 9 10 11
12 13 14
NH
-Ile-Val-Gly-Gly-XXX-Glu-XXX-Glu-Gln-His-Ser-Gln-Pro
-XXX-
30 15 16
Gln-Ala-----,

wherein XXX denotes residues that cannot be positively assigned. The result indicated that the N-terminal sequence of recombinant kallikrein is
35 identical to the previously determined N-terminal sequence of human urinary kallikrein.

N-terminal sequence analysis of recombinant prokallikrein revealed that the proform contains a heptapeptide leader followed by the amino acid sequence of mature kallikrein, i.e.,

1 2 3 4 5 6 7 8 9 10 11 12
13 14
Ala-Pro-Pro-Ile-Gln-Ser-Arg-Ile-Val-Gly-Gly-XXX-Glu-
10 XXX-
15 16 17 18 19 20
GLU-Gln-His-Ser-Gln-Pro-----,

where XXX denotes residues that cannot be positively assigned. This proform N-terminal sequence is also identical to the N-terminal sequence reported by Takahashi et al., supra.

Example 14

20 Kinongenase Assay for Kallikrein

The procedure used for measuring the kinin-generating activity of the recombinant kallikrein polypeptide of the present invention was essentially the same as the described by Shimamoto et al., Jap. Circ. J. 43: 147-152), (1979):

Purified human urinary kallikrein standards or recombinant kallikrein samples diluted (20 μ l or 40 μ l) in 0.1 M sodium phosphate (pH 8.5)/30 mM Na₂EDTA/3 mM phenanthroline containing 3 μ g of purified bovine low molecular weight kininogen in a total volume of 0.5 ml were incubated at 37°C for 30 min. The reaction was terminated by boiling for 10 min. and 50 μ l aliquots in duplicate were used to measure the amount of kinin-released by a kinin RIA

with a rabbit antiserum against kinin. The
kininogenase activity of recombinant kallikrein is
expressed as the amount of generated kinin in $\mu\text{g}/30$
min/mg kallikrein.

10

Example 15

In Vivo Assay for Blood Pressure-Lowering Effect of Kallikrein

A. Rats

Male species of spontaneous hypertensive rats, about
250 grams body weight, were used in the study. Rats
were anesthetized with sodium pentobarbital (50mg/Kg
body weight) intraperitoneally. The common carotid
artery was cannulated and then connected to a
Statham pressure transducer. The right jugular vein
was cannulated for administering kallikrein and
changes in blood pressure were measured with a
polygraph. Upon administration of the recombinant
mature kallikrein of the present invention, lowering
blood pressure levels in the hypertensive rats was
observed.

B. Pigs

In order to carry out conscious animal experiments
for the drug test the following protocol was used.
Normal farm pigs weighing approximately 50kg, were

sedated with 25 mg ketamine HCl, per Kg, IM, and anesthesia was induced with 20 mg sodium thiamylol per Kg, IV and was maintained with 1-2% halothane. A left thoracotomy was performed at the fourth or fifth rib space. Both an aortic and left atrial catheter were implanted and exteriorized through the back.

The animals were allowed to recover one week before undergoing an experiment. During the recovery period each animal was trained for the drug testing procedure. All animals were monitored daily to insure good health.

The animals were loaded into the experimental cart and the two catheters of each animal were flushed. The aortic line was used to continuously monitor pressure. During each experiment the animal received 10,000 U heparin/hour to prevent clot formation. Once a stable blood pressure was reached i.e., mean arterial pressure was maintained ± 5 mmHg for five minutes, the test began. The drug was then administered through the left atrial catheter with continual pressure monitoring. The time interval between drug tests was dependent on the animal and the dose previously given. The two preparations of pure recombinant kallikrein (rHK) designated as K-011 and K-012 were used in the tests. For K-011, assuming 1 mg rHK = 1.5 A₂₈₀, 0.25 ml solution used in the test is equivalent to the dosage of 2.5 μ g rHK/Kg body weight, and for preparation K-012, 0.25 ml solution is equivalent to 1.25 μ g rHK/Kg body weight.

The results are shown below in Tables XI - XIV.
Even at a concentration as low as 1.25 µg/kg body
weight can cause significant reduction in mean
atrial pressure in the conscious pig (see Table XIV).

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TABLE XI
DRUG K-011
FIG 500

5	dose=0.25 ml				dose=0.50 ml			
	Time	HR	BP	MAP	Time	HR	BP	MAP
10	cont.	115	170/115	137	cont.	105	165/115	132
	30 sec	210	175/100	125	30 sec	165	140/70	93
	1min	175	175/100	125	1min	147	155/70	98
	2 min	160	175/115	137	2 min	125	160/110	127
	3 min	145	175/115	137	3 min	120	160/110	127
15	4 min	130	175/115	137	4 min	120	160/110	127
	5 min	115	175/115	137	5 min	120	160/110	127
	6 min	105	175/115	137	6 min	120	160/110	127
20	dose=1.0 ml				dose=2.0ml			
	Time	HR	BP	MAP	Time	HR	BP	MAP
25	cont.	120	190/145	163	cont.	165	165/115	132
	30 sec	165	115/75	88	30 sec	180	115/75	88
	1 min	165	155/70	127	1 min	165	160/105	123
	2 min	160	160/110	117	2 min	165	160/105	123
	3 min	165	165/115	132	3 min	165	160/115	130
	4 min	165	165/115	132	4 min	165	160/115	130
30	5 min	165	165/115	132	5 min	165	160/115	130
	6 min	165	165/115	132	6 min	165	160/115	130

cont. = control

HR = heart rate

BP = blood pressure

MAP = mean atrial pressure

TABLE XII
DRUG K-011
FIG 14

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dose=0.25 ml					dose=0.50 ml			
	Time	HR	BP	MAP	Time	HR	BP	MAP
10	cont.	96	115/65	82	cont.	81	100/60	73
	30 sec	167	100/30	53	30 sec	120	100/30	53
	1 min	120	100/30	53	1 min	138	115/40	65
	2 min	96	100/35	55	2 min	108	90/30	50
15	3 min	90	100/45	63	3 min	84	85/40	55
	4 min	90	110/60	77	4 min	78	95/50	65
	5 min	78	100/65	77	5 min	72	95/60	72
	6 min	80	105/75	85	6 min	72	95/60	72
dose=1.0 ml					dose=2.0ml			
	Time	HR	BP	MAP	Time	HR	BP	MAP
20	cont.	90	100/60	163	cont.	72	95/60	72
	30 sec	138	95/35	88	30 sec	138	80/25	43
	1 min	120	100/40	127	1 min	120	100/45	63
	2 min	96	100/50	117	2 min	96	95/55	68
25	3 min	90	100/60	132	3 min	96	100/60	73
	4 min	90	90/55	132	4 min	84	100/70	80
	5 min	78	95/60	132	5 min	90	100/70	80
	6 min	78	95/60	132	6 min	72	105/70	82
30								

cont. = control

HR = heart rate

BP = blood pressure

MAP = mean atrial pressure

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TABLE XIII
 DRUG K-012
 FIG 14

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dose=0.25 ml					dose=0.50 ml				
	Time	HR	BP	MAP		Time	HR	BP	MAP
10	cont.	96	115/75	88		cont.	114	135/95	108
	30 sec	132	100/40	60		30 sec	204	110/55	73
	1 min	115	105/65	78		1 min	132	110/60	77
	2 min	104	110/75	86		2 min	114	105/70	81
15	3 min	104	110/75	86		3 min	102	110/75	86
	4 min	104	110/75	86		4 min	102	110/75	86
	5 min	104	110/75	86		5 min	102	110/75	86
	6 min	104	110/75	86		6 min	102	110/75	86
20	dose=1.0 ml								
	Time	HR	BP	MAP					
	cont.	96	110/75	86					
25	30 sec	168	100/40	60					
	1 min	136	100/42.5	61.5					
	2 min	102	100/50	67					
	3 min	96	100/55	70					
	4 min	90	100/65	77					
30	5 min	84	100/70	80					
	6 min	84	100/70	80					

cont. = control

HR = heart rate

BP = blood pressure

MAP = mean atrial pressure

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TABLE XIV
DRUG K-012
PIG 43

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	dose=2.0				dose=4.0			
10	Time	HR	BP	MAP	Time	HR	BP	MAP
	cont.	132	145/75	98	cont.	96	125/85	98
	30 sec	250	100/35	57	30 sec	114	115/45	68
	1min	216	95/45	62	1min	102	100/35	57
15	2 min	144	90/50	63	2 min	96	110/55	73
	3 min	120	90/60	70	3 min	84	110/75	86
	4 min	120	120/85	97	4 min	90	110/75	86
	5 min	96	125/85	98	5 min	84	115/85	95
	6 min	96	135/100	112	6 min	78	130/100	110

20

cont. = control

HR = heart rate

BP = blood pressure

25 MAP = mean atrial pressure

The longest response obtained was six minutes.

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Example 16Kininogenase Activity and Esterase Activity of
Purified Recombinant Human Kallikrein

Recombinant human kallikrein acted on the substrate kininogen to release kinin, i.e., kininogenase activity, an activity associated with naturally-occurring kallikrein. As represented in Table XV, the activity of the purified recombinant human mature kallikrein of the present invention was similar to the activity obtained from purified human urinary kallikrein.

Table XV

	Kininogenase Specific Activity (1)	Esterase Unit (2)
5		
	Recombinant kallikrein	29.0
	Human urinary kallikrein	28.9
	Buffer alone	0
10		

(1) Kininogenase specific activity is expressed as
µg kinin generated/30 min/mg kallikrein and is
determined in accordance with the procedures of
Example 12.

(2) Esterase unit is measured using: 3H- Tbs-Arg-OMe
as substrate and expressed as E.U./mg kallikrein
and is determined in accordance with the
procedures of Beaven et al., [Clin. Chim. Acta.
32, 67 (1971)].

The recombinant human kallikrein exhibited a dose
response curve parallel to that of the purified
human urinary kallikrein as measured by
radioimmunoassay procedure of Example 7. This
result indicates that the kallikrein produced by the
recombinant cells have the same immunological
property as naturally-occurring kallikrein produced
by native cells.

The purified recombinant human mature kallikrein was
analyzed in a 8-25% gradient sodium dodecyl sulfate-
polyacrylamide gel and both recombinant human mature
kallikrein, and naturally-occurring urinary
kallikrein, have similar molecular weight a

nd both showed heterogeneity in size (Figure 2).
Size heterogeneity is common for glycoproteins due
to the variation in the sugar chain length.

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Example 17

Effect of Recombinant Human Kallikrein on Human
Sperm Motility

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Sperm were obtained from fertile normal donors.
Purified human kallikrein (0.68 A₂₈₀) was added at
the final dilution of 1:10 and 1:20 to 1-2x10⁶
sperms in 100 µl final volume.

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Sperm motility was measured using the procedure
described by Mathur et al., Fertility and Sterility,
Vol. 46, No. 3, 484(1986) and Mathur et al.,
American Journal of Reproductive Immunology and
Microbiology, 12:87-89(1986) hereby incorporated by
reference. The results from a 48hr observation were
as follows:

20

	<u>% Motile</u>
Control Media	29.13
25 r-HuK (1:10 dilution)	42.76
r-HuK (1:20 dilution)	41.26

The recombinant kallikrein significantly enhanced
the sperm motility. This biological activity of
recombinant kallikrein is important as a therapeutic
agent for treating male infertility.

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* * *

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Numerous modifications and variations in the
practice of the invention are expected to occur to
those skilled in the art upon consideration of the
foregoing illustrative examples. Consequently, the

invention should be considered as limited only to
the extent reflected by the appended claims.

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WHAT IS CLAIMED IS:

1. A purified and isolated recombinantly derived kallikrein polypeptide comprising the amino acid sequence, extending from the N-terminus:

(Ala Pro Pro Ile Gln Ser Arg⁻¹)_n

+1	10	20
Ile Val Gly Gly Trp Glu Cys Glu Gln His Ser Gln Pro Trp Gln Ala Ala Leu Tyr His		
	30	40
Phe Ser Thr Phe Gln Cys Gly Gly Ile Leu Val His Arg Gln Trp Val Leu Thr Ala Ala		
	50	60
His Cys Ile Ser Asp Asn Tyr Gln Leu Trp Leu Gly Arg His Asn Leu Phe Asp Asp Glu		
	70	80
Asn Thr Ala Gln Phe Val His Val Ser Glu Ser Phe Pro His Pro Gly Phe Asn Met Ser		
	90	100
Leu Leu Glu Asn His Thr Arg Gln Ala Asp Glu Asp Tyr Ser His Asp Leu Met Leu Leu		
	110	120
Arg Leu Thr Glu Pro Ala Asp Thr Ile Thr Asp Ala Val Lys Val Val Glu Leu Pro Thr		
	130	140
Gln Glu Pro Glu Val Gly Ser Thr Cys Leu Ala Ser Gly Trp Gly Ser Ile Glu Pro Glu		
	150	160
Asn Phe Ser Phe Pro Asp Asp Leu Gln Cys Val Asp Leu Lys Ile Leu Pro Asn Asp Glu		
	170	180
Cys Lys Lys Ala His Val Gln Lys Val Thr Asp Phe Met Leu Cys Val Gly His Leu Glu		
	190	200
Gly Gly Lys Asp Thr Cys Val Gly Asp Ser Gly Gly Pro Leu Met Cys Asp Gly Val Leu		
	210	220
Gln Gly Val Thr Ser Trp Gly Tyr Val Pro Cys Gly Thr Pro Asn Lys Pro Ser Val Ala		
	230	238
Val Arg Val Leu Ser Tyr Val Lys Trp Ile Glu Asp Thr Ile Ala Glu Asn Ser		

wherein n is 0 or 1; and characterized by being the product of procaryotic or eucaryotic expression of an exogenous DNA sequence.

5

2. A purified and isolated recombinantly derived kallikrein polypeptide according to Claim 1 wherein n is 0.

10

3. A purified and isolated recombinantly derived kallikrein polypeptide according to Claim 1 wherein n is 1.

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4. A purified and isolated recombinantly derived kallikrein polypeptide according to Claim 2 and free of association with any mammalian protein.

20

5. A purified and isolated recombinantly derived kallikrein polypeptide according to Claim 2 wherein the exogenous DNA sequence is a cDNA sequence.

25

6. A purified and isolated recombinantly derived kallikrein polypeptide according to Claim 2 wherein the exogenous DNA sequence is a manufactured DNA sequence.

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7. A purified and isolated recombinantly derived kallikrein polypeptide according to Claim 2 wherein the exogenous DNA sequence is a genomic DNA sequence.

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8. A purified and isolated recombinantly derived kallikrein polypeptide according to Claim 1 having a detectable label.

9. A purified and isolated DNA encoding for procaryotic or eucaryotic host expression of a kallikrein polypeptide of Claim 1.
- 5 10. A purified and isolated DNA according to Claim 9 having the nucleotide sequence set forth in Table V.
- 10 11. A purified and isolated DNA according to Claim 9 wherein the DNA is cDNA.
12. A purified and isolated DNA according to Claim 9 wherein the DNA is genomic DNA.
- 15 13. A purified and isolated DNA according to Claim 9 wherein the DNA is manufactured DNA.
- 20 14. A purified and isolated DNA according to Claim 13 having one or more codons preferred for expression in E. coli cells.
- 25 15. A purified and isolated DNA according to Claim 14 having the nucleotide sequence set forth in Table VI.
- 30 16. A pharmaceutical composition comprising a therapeutically effective amount of a kallikrein polypeptide of Claim 1 and pharmaceutically acceptable adjuvants.
- 35 17. A method for providing vasodilation therapy comprising administering a therapeutically effective amount of a kallikrein polypeptide according to Claim 1.
18. A method of treating male infertility comprising administering a therapeutically effective amount

of a kallikrein polypeptide according to
Claim 1.

- 5 19. A procaryotic or eucaryotic host cell
transformed or transfected with DNA according to
Claim 9 in a manner allowing the host cell to
express a kallikrein polypeptide product of
Claim 1.
- 10 20. A plasmid selected from the group consisting of
pDHSK11 and pDGHK-L1A.
- 15 21. A eucaryotic host cell transformed or
trasfected with a plasmid of claim 20.
22. A method of producing a purified and isolated
kallikrein polypeptide comprising the steps of:
transfecting or transforming host cells
20 with DNA according to claim 9;
culturing the transfected or transformed
host cells to allow the host cells to express
kallikrein polypeptide; and
isolating kallikrein.
- 25 23. A method for the purification of recombinant
human kallikrein comprising the steps of:
concentrating culture medium containing
recombinant human kallikrein by diafiltration;
30 and
subjecting the culture medium to affinity
chromatography.
- 35 24. A method as in claim 23 wherein after the
concentrating step is the step of subjecting the
culture medium to anion exchange chromatography,
and after the affinity chromatography step is

the step of subjecting the culture medium to gel
filtration.

- 5 25. A method as in claim 24 wherein after the anion
exchange chromatography step is the step of
converting prokallikrein to kallikrein by
autoactivation.

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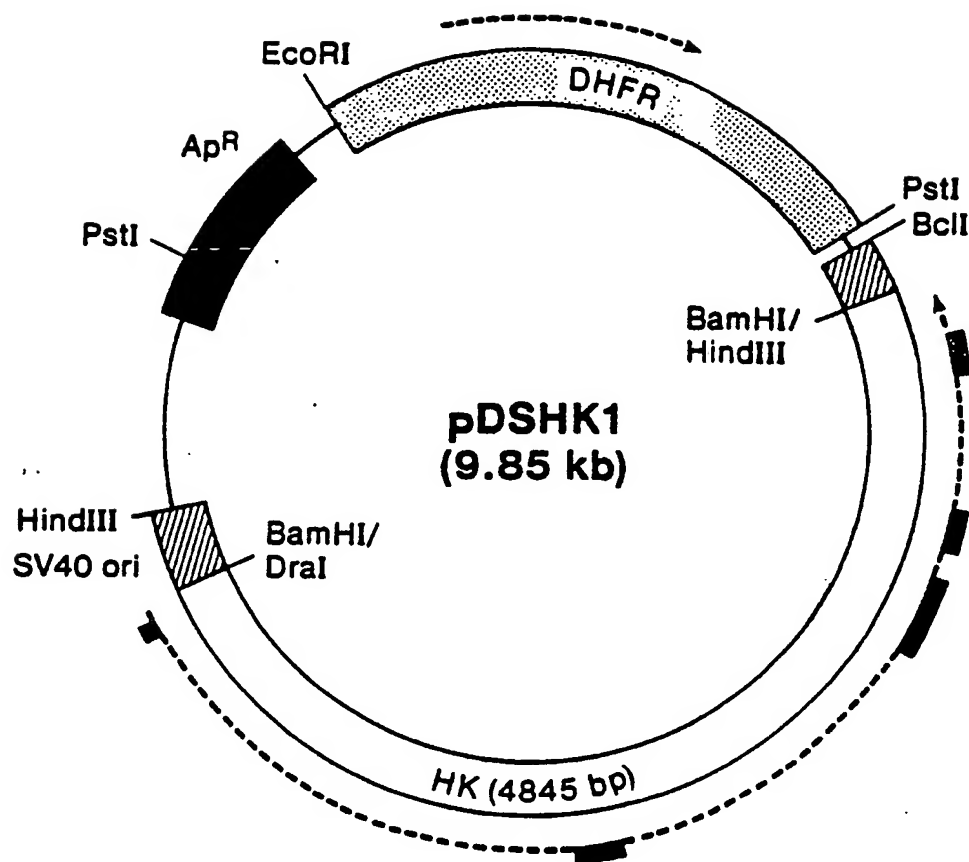


FIG.1

- 2 / 3 -

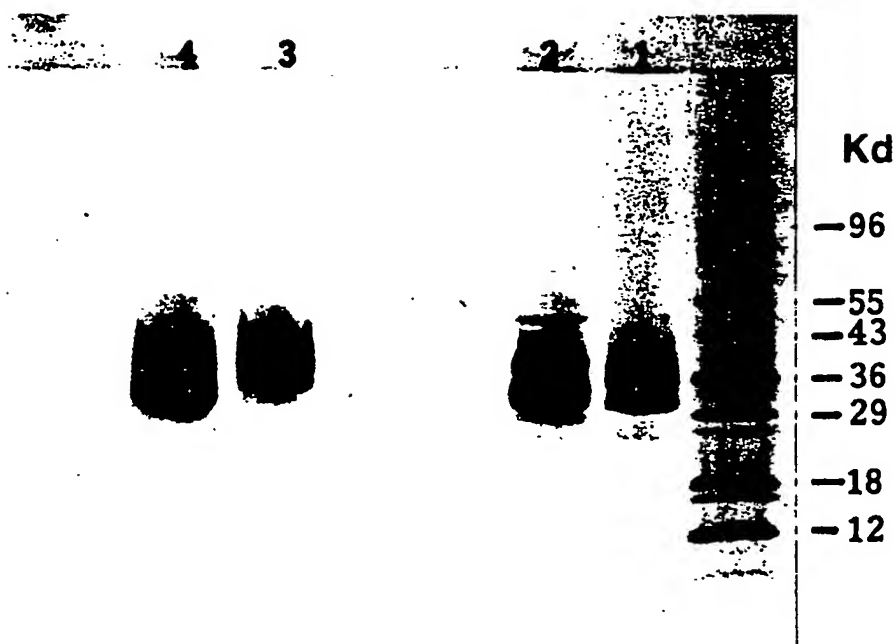


FIG.2

SUBSTITUTE SHEET

- 3 / 3 -

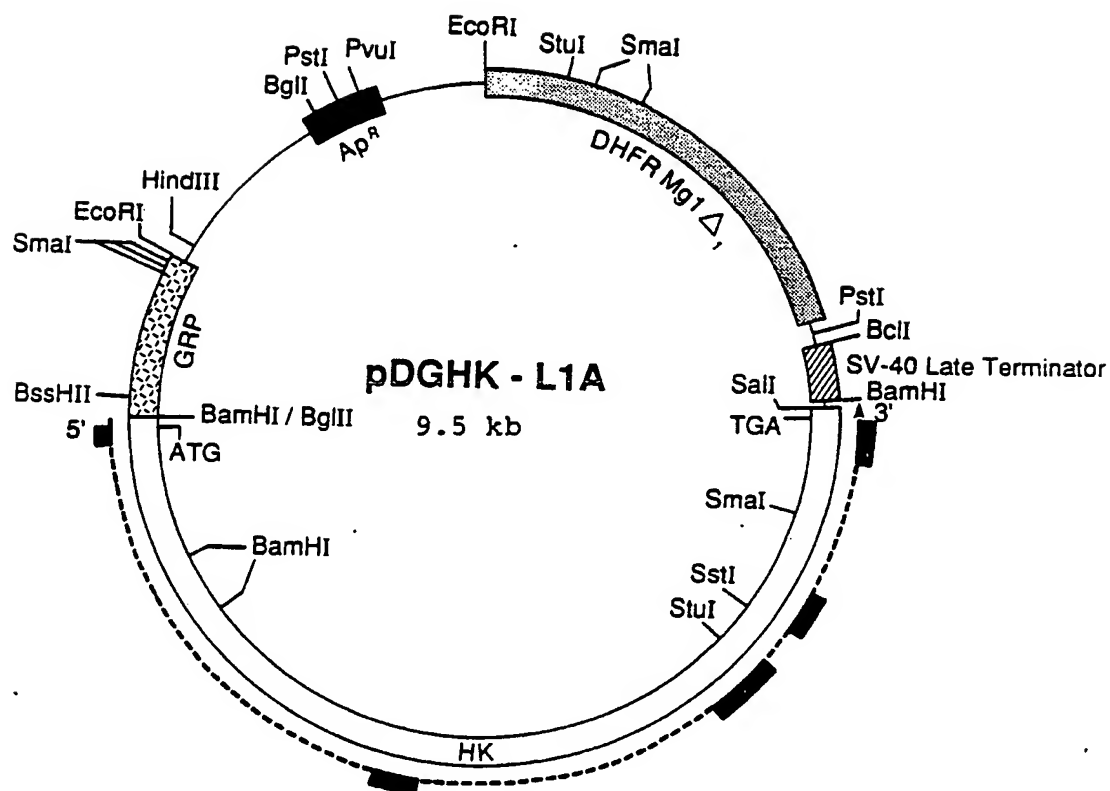


FIG.3

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US88/02214**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C12N 9/64, C12N 15/00, C12N 1/20, C12N 5/00, A61K 37/553, C07H 21/00 US CL 435/226, 253, 240.2, 172.3, 320, 424/94.64 536/27											
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; text-align: left; padding: 5px;">Classification System</th> <th style="border: 1px solid black; text-align: left; padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">U.S.</td> <td style="border: 1px solid black; padding: 5px;">435/172.3, 226, 240.2, 253, 320 935/14, 29, 32, 70, 72 424/94.64, 99 536/27</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> <p>Chemical Abstract Data Base (CAS) 1967-1988, BIOSIS DATA BASE (1969-1988) Keywords: Kallikrein, Plasmid, vector, recombinant, human, monkey, express?, sequence search on Intelligenetics and protein identification resource Data Base.</p>			Classification System	Classification Symbols	U.S.	435/172.3, 226, 240.2, 253, 320 935/14, 29, 32, 70, 72 424/94.64, 99 536/27					
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents. ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>											
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">22 September 1988</td> <td style="border: 1px solid black; padding: 5px;">15 NOV 1988</td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">ISA/US</td> <td style="border: 1px solid black; padding: 5px;"> JAYME A. HULEATT </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	22 September 1988	15 NOV 1988	International Searching Authority	Signature of Authorized Officer	ISA/US	 JAYME A. HULEATT	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y, P	<u>DNA</u> , Volume 6, Number 5, issued 1987, October (New York, New York, U.S.A.) (L. SCHEDLICH ET AL.), "Primary Structure of a Human Glandular Kallikrein Gene". See pages 429-437, see particularly pages 429, 430 and 432 and 434.	1-25
Y	<u>Hoppe-Seyler's Z. Physiol. Chem.</u> Volume 360, issued 1979, December (Berlin, West Germany), (F. LOTTSPREICH ET AL.), "N-Terminal Amino Acid Sequence of Human Urinary Kallikrein Homology with other Serine Proteases". See pages 1947-1950, see particularly pages 1947 and 1949.	1-25
Y	<u>Nucleic Acids Research</u> , Volume 14, Number 12, issued 1986, June (Oxford, England), (M. FAHNESTOCK ET AL.) "The sequence of a cDNA clone coding for a novel kallikrein from mouse submaxillary gland". See pages 4823-4835, see particularly pages 4830-4831.	1-25
X Y	U.S., A, 4,510,248 (NAKANISHI ET AL) 9 April 1985. See abstract and column 2.	1-7, 16, 17 8, 18 and 23-25
X Y	U.S., A, 3,905,870 (KUTZBACH ET AL.) 16 September 1975 (See abstract and columns 3, 4, 5 and 6	1-7, 16 8, 17, 18 and 23-25
Y	<u>Chemical Abstracts</u> , Volume 106, Number 19, issued 1987, May 11 (Columbus, Ohio, U.S.A.), (B. SOMLEV ET AL.), "Stimulation of the mobility of bull spermatozoa after freezing using kallikrein and bradykinin", see page 87, columns 1 and 2, the abstract No. 149724d. Dokl. Bolg. Akad. Nauk. 1986, 39(11), 115-118 (Russ.)."	18

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

PCT/US88/02214

Attachment to PCT/ISA/210
Observations Where Unity of Invention Is Lacking

Group I includes claims 1-8, 16 and 17, drawn to kallikrein, a pharmaceutical composition containing kallikrein and a method of administering kallikrein, for treating vasodilation therapy classified in 435/226 and 424/94.64.

Group II includes claim 18 which is a second method of administering kallikrein for treating male infertility, classified in 424/94.64.

Group III includes claims 9-15 and 19-22 drawn to DNA encoding for kallikrein, host cells transformed with the DNA, a plasmid containing the DNA and a method of using the transformed host cells to make kallikrein classified in 536/27 and 435/226, 253, 240.2, 255 and 320.

Group IV includes claims 23-25 drawn to a method of purifying kallikrein by chromatography classified in 435/226.